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Effects of *in Utero* Exposure to Arsenic during the Second Half of Gestation on Reproductive End Points and Metabolic Parameters in Female CD-1 Mice

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Running title: Effects of *in utero* arsenic in female mice

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Abstract

Background: Mice exposed to high levels of arsenic *in utero* are more susceptible to tumors such as hepatic and pulmonary carcinoma when they reach adulthood. However, effects of *in utero* arsenic exposure on general physiological functions such as reproduction and metabolism remain unclear.

Objectives: We evaluated the effect of *in utero* exposure to inorganic arsenic at the EPA drinking water standard (10 ppb) and tumor-inducing level (42.5 ppm) on reproductive end points and metabolic parameters when the exposed females reach adulthood.

Methods: Pregnant CD-1 mice were exposed to sodium arsenite (0, 10 ppb, or 42.5 ppm) in drinking water from gestational day 10 to birth, the window of organ formation. At birth, exposed offspring were fostered to unexposed dams. We examined reproductive end points (age at vaginal opening, reproductive hormone levels, estrous cyclicity, and fertility) and metabolic parameters (body weight changes, hormone levels, body fat content, and glucose tolerance) of the exposed females in adulthood.

Results: Arsenic-exposed females (10 ppb and 42.5 ppm) exhibited early onset of vaginal opening. Fertility was not affected when females were exposed to the 10 ppb dose. However, the number of litters per female was decreased in females exposed to 42.5 ppm of arsenic *in utero*. In both 10 ppb and 42.5 ppm groups, exposed females had significantly higher body weight gain, body fat content, and glucose intolerance.

Conclusion: Our findings reveal unexpected effects that *in utero* exposure to arsenic at a human relevant low dose and a tumor-inducing level leads to early onset of vaginal opening and obesity in female CD-1 mice.

Introduction

Developmental origins of adult disease are implicated in cardiovascular diseases (Barker et al. 1989; Forsen et al. 1999), diabetes (Fall et al. 1998; Vignini et al. 2012), cancers, and reproductive disorders such as polycystic ovarian syndrome (Xita and Tsatsoulis 2010; Xu et al. 2014). The nutritional and physical status of the mothers and their exposure to various environmental toxicants during pregnancy are contributing factors to the fetuses' susceptibility to various diseases when the fetuses reach adulthood (reviewed in (Boekelheide et al. 2012). One such environmental toxicant is inorganic arsenic. Arsenic is a metalloid naturally found in the environment and a common contaminant in the drinking water (Smedley and Kinniburgh 2002) and in crops such as rice (Charnley 2014). In the United States, the maximum contaminant level of arsenic in the drinking water set by the EPA is 10 ppb (part per billion; EPA-816-K-02-018). Many private wells in the US and groundwater in other parts of the world have levels above 10 ppb (up to > 5000 ppb; reviewed (Smedley and Kinniburgh 2002).

Gestation is a sensitive period for arsenic toxicity (Devesa et al. 2006; Kozul-Horvath et al. 2012). In humans, chronic exposure to inorganic arsenic was linked to cardiovascular disease, diabetes mellitus and cancers of the skin, lung, liver, urinary bladder and prostate (Brauner et al. 2014; Moon et al. 2013; Smith et al. 2013; Steinmaus et al. 2014). In mice, *in utero* exposure to arsenic (in doses ranging from 42.5 ppm to 85 ppm) resulted in an increased incidence of lung, liver, adrenal, skin and ovarian tumors when the exposed embryos reach adulthood (Liu et al. 2007; Tokar et al. 2010; Waalkes et al. 2004b). The urogenital system is a known target tissue for arsenic toxicity as CD-1 mice exposed to arsenic at 85 ppm *in utero* from embryonic day or E8 to 18 exhibited increased incidence of ovary, uterus and adrenal gland tumors at 90 weeks of age (Waalkes et al. 2006).

Although the detrimental impacts of high level arsenic exposure (ppm range) are well documented, it is not clear what the consequence of exposure to levels relevant to normal human consumption (ppb range) may be. Exposure to 50 ppb arsenic from fetal life to adulthood increased lung tumor incidence in female CD-1 mice (Waalkes et al. 2014), while exposure to 10 ppb arsenic during pregnancy resulted in liver steatosis and decreased breast milk triglyceride levels in exposed C57BL6/J dams, leading to growth deficits in their offspring (Kozul-Horvath et al. 2012). In this study, we investigated the effects of 10 ppb arsenic (the maximum contaminant level in drinking water, MCL, designated by the EPA, 66 FR 6976) for its relevance to human exposure. We also exposed the mice to 42.5 ppm arsenic in the drinking water to define the impact of *in utero* arsenic exposure at a known tumor-inducing level (Tokar et al. 2010; Waalkes et al. 2003) on general physiological functions from puberty to 1 year of age. The exposure period was restricted to the second half of gestation from E10 to birth (critical window of fetal organ formation in mice) and the animals were allowed to develop to adulthood without further exposure. We focus on reproductive and metabolic endpoints, which are known to have physiological interactions.

Materials and Methods

Animals and treatments

Female CD-1 mice at the age of 8-10 weeks (Charles River, Wilmington, MA) were timed-mated with CD-1 males. The day that the vaginal plug was detected was considered as embryonic day 0 or E0 and the pregnant females were housed individually in plastic cages using Sani Chips bedding (P.J. Murphy Forest Products Corp.). The pregnant females were provided *at libitum* with NIH-31 chow and water processed through a reverse osmosis deionized system. Arsenic was below the detection level in the NIH-31 chow (analyzed by inductively coupled

plasma atomic emission spectroscopy; Microbac Laboratories). At E10, pregnant females were randomly assigned to one of the following treatment groups (11 pregnant females per group): 1) Control, no inorganic arsenic; 2) 10 ppb inorganic arsenic (as Sodium arsenite; Spectrum Chemicals, New Brunswick, NJ); or 3) 42.5 ppm inorganic arsenic in the drinking water. The treatment window was from E10 to birth. Pregnant females were allowed to deliver naturally and newborn pups were immediately fostered to females that were not exposed to arsenic. In order to ensure even growth of the pups, each foster female were given 10 newborns from the same litter. Female pups from each litter were assigned to experiments listed in Table 1. The timeline of the experiments is outlined in Figure 1. All animals were maintained in standard plastic mouse cages (maximum of 5 mice per cage), in temperature controlled rooms, and under controlled lighting (12L:12D). Euthanasia was performed by CO2 inhalation. All animal procedures were approved by the National Institutes of Health Animals Care and Use Committee and were performed in accordance with an approved National Institute of Environmental Health Sciences animal study proposal. All animals were treated humanely with regard to alleviation of suffering.

Vaginal opening and estrous cyclicity analysis

Female pups (n=29 for control; n=37 for 10 ppb group, n=35 for 42.5 ppm group) were checked at 9AM daily for status of the vaginal opening starting at 18 days of age until the day that all females exhibited an open vaginal canal. Estrous cycle was monitored in females starting at 10 weeks of age by vaginal smears taken daily (9 AM) in their cage for 18 consecutive days. Vaginal smears were immediately fixed on glass slides (Safetex; Andwin Scientific) and stained with hematoxylin and eosin following standard H&E protocols. Phases of the estrous cycle were determined based on vaginal cytology as previously described (Jayes et al. 2014)

Fertility Study

When females reached 8 weeks of age, they were placed individually in a continuing mating scheme. Breeding pairs of females (Control: n=5; 10 ppb: n=8; 42.5 ppm: n=13) and proven, not exposed males (CD-1 males, 10-12 weeks old) were housed together (one pair per cage) until they reach one year of age. The parameters analyzed during the 1-year period included: days to first litter, days between litters, average number of pups per litter, total number of pups produced per female, total number of litters per female, and fertile period (measured as the number of days from initial mating to the last litter).

Measurement of Body weight and body fat composition

Mice were weighed weekly starting at weaning (21 days) until 15 weeks of age. Weights of mice in the fertility study were included only prior to the beginning of the breeding period The sample size for body weight measurement up to 8 weeks of age were: 29 for the control, 37 for the 10 ppb group, 35 for the 42.5 ppm group. The sample sizes for the body weight analysis from 8 to 15 weeks of age were: 17 for the control, 25 for the 10 ppb group, and 21 for the 42.5 ppm group. Body fat composition was analyzed by using PIXImus® densitometer (GE Lunar Corporation; Waukesha, WI) at 4.5 months of age in mice not included in the fertility study (n=9 for the control, n=10 for the 10 ppb group; n=11 for the 42.5 ppm group).

Serum analysis

Hormone analysis was performed in serum collected at different ages from females that were not included in the fertility study. Following euthanasia, blood was collected by either cardiac puncture or from the descending vena cava. Serum (collected from non-fasted females) was separated using BD Microtainer™ Plastic Capillary Blood Collectors (BD Diagnostics,

Franklin Lakes, NJ) and frozen in -80C. Serum from 21 and 28 days old females was used to measure the levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by using the Milliplex Map Mouse Pituitary Magnetic Bead Panel (Cat. MPTMAG-49K; Millipore, Billerica, MA; control n=29; 10 ppb n=37; 42.5 ppm n=35). Serum from 6 months old females was examined for the levels of leptin and insulin by using the Mouse Metabolic Kit (cat # N45124A-1) from MSD (Meso Scale Discovery, Gaithersburg, Maryland, USA) following manufacturer's protocols (control n=5; 10 ppb n=7; 42.5 ppm n=6). Serum from 21 days, 28 days, 6 months and 1 year old females was examined for the levels of estradiol, dehydroepiandrosterone (DHEA), testosterone and progesterone by using the Multi Spot 96 HB 4-Spot Custom Steroid Hormone Panel (Cat. N45CB-1; Meso Scale Diagnostics, Rockville, MD. Data is presented in Supplemental Figure S3 (control n=5; 10 ppb n=5; 42.5 ppm =6). All samples were assayed in duplicate.

Glucose tolerance test

Five months old females that were not included in the fertility study (control n=7; 10 ppb n=10; 42.5 ppm n=11) were fasted overnight and their baseline glucose levels in the serum were determined by novaMaxPlus glucometer (Nova Biomedical, Waltham, MA). Mice were then given an intraperitoneal injection of D-glucose (2 mg/g body weight) and blood samples were collected for glucose measurement at 20, 40, 60, 120 and 180 min after the injection.

Additional endpoints

The body weight analysis for the E18 embryos is presented in Supplemental Figure S1 (control n=46; 10 ppb n=30; 42.5 ppm n=38). The ovaries from animals collected at postnatal day 21 and 28 and 6 months of age (control n=5-7; 10 ppb n=5-8; 42.5 ppm n=6-8 for each time

point) were fixed overnight in PFA and stained with hematoxylin and eosin (H&E). Results are shown in Supplemental Figure S2.

Statistical Analysis

Sample size for each experiment is listed in Table 1. Data on age at vaginal opening was analyzed using log-rank statistics and mixed model analysis of covariance adjusting for weaning weight, dam and litter effects. For the estrous cyclicity data, the percentage of time spent in each of the four stages (estrus, metestrus, diestrus, or proestrus) among the treatment groups was compared using mixed effects analysis of variance, with dam as a random effect to take correlations into account. Dunnett's test was used to compare each treatment group to the control group. Body weight and body fat composition were analyzed using a mixed model ANOVA using dam as a random effect to take littermate correlation into consideration. Hormonal levels and fertility data were compared using ANOVA and Tukey's multiple comparison tests. P values less than 0.05 were considered statistically different.

Results

The goal of the study was to investigate how *in utero* exposure to 10 ppb (EPA MCL in drinking water) and tumor-inducing (42.5 ppm; (Waalkes et al. 2003; Waalkes et al. 2004b) levels of arsenic in drinking water affects reproductive and metabolic functions when the exposed animals reach adulthood. We restricted the exposure period to the second half of gestation to investigate specifically the impact of arsenic on organ formation. Pregnant CD-1 females exposed to arsenic showed no effects of treatment on body weight gain and number of pups born per litter (Figure S1). The weights of the fetuses at E18 were similar among treatment groups with the exception of 10 ppb group, which had a significant increase in body weight

(Figure S1). The pups born from exposed females appeared healthy without any signs of stress or malformation and were able to develop to adulthood for analyses of reproductive and metabolic endpoints.

Impacts of in utero arsenic exposure on reproductive functions

One of the first signs of reproductive development in female mice is the opening of the vagina, an external indicator of the onset of puberty (Hansen et al. 1983). Vaginal opening was first detected in control females at 23 days of age and by 30 days, all control females exhibited open vagina (Figure 2A). In contrast, pups exposed to arsenic *in utero* (both 10 ppb and 42.5 ppm groups) exhibited vaginal opening as early as 21 days (Figure 2A). Compared to control with 26.5 ± 0.3 days of mean age at vaginal opening, the arsenic-exposed females had significantly early onset of vaginal opening (23.8 ± 0.2 days for 10 ppb group and 24.5 ± 0.3 days for 42.5 ppm group in Figure 2B). Onset of vaginal opening in mice is known to positively associate with body weight (Hansen et al. 1983). The pups exposed to either 10 ppb or 42.5 ppm *in utero* displayed higher body weights at weaning (21 days of age) compared to controls ($p< 0.001$; Figure 2C). Significant negative correlations between body weight and age at vaginal opening were detected in controls (Figure 2D, $r^2= -0.51$; $p=0.004$) and 42.5 ppm group ($r^2=-0.62$; $p<0.001$). However, no significant correlation was observed in the 10 ppb group ($r^2= 0.032$; $p=0.85$), suggesting that exposure to 10 ppb *in utero* causes early onset of vaginal opening independent of body weight.

We next examined the level of gonadotropins (LH and FSH), pituitary-derived hormones that trigger reproductive development of the females. At 21 days, serum level of LH was significantly elevated in females exposed to 10 ppb of arsenic *in utero* compared to the controls

and this effect is not observed at 28 days (Figure 3A & 3B), whereas no differences was detected in females exposed to 42.5 ppm arsenic *in utero* at either 21 or 28 days of age. Serum FSH level was not significantly different between the control and treatment groups at either time point (Figure 3C & 3D).

Progression of the estrous cycle is another endpoint indicative of proper reproductive development. We monitored the estrous cycle in exposed females for 18 consecutive days (roughly 3-4 cycles) when they reached 2.5 months of age. The percentage of the time that the female spent in each stage of the estrous cycle (P: Proestrus, E: Estrus, M: Metestrus, and D: Diestrus) was not statistically different among the control and treatment groups (Table 2).

To test whether fertility was affected by *in utero* exposure to arsenic, females were housed with fertile CD-1 males from 8 weeks to 1 year of age, and examined for fertility outcomes. We found no differences between the control and treatment groups on number of days to first litter, average days between litters, and average number of pups per litter (Table 3). While no differences were found between the control and treatment groups in the total number of litters born per female, we detected a significant difference between the 10 ppb and 42.5 ppm groups with less litters born, less pups per female, less total number of pups born and a shorter fertile period (measured as the number of days from initial mating to the last litter) in the 42.5 ppm exposed group compared to the 10 ppb exposed group (Table 3). No detectable differences were observed in ovarian morphology at 21 days, 28 days and 6 months of age between control and *in utero* exposed females (Figure S2). Although some changes were detected in the pattern of circulating sex steroid levels at 6 month and 1 year of age (Figure S3), these changes were not statistically significant. In summary, *in utero* exposure to arsenic at either 10 ppb or 42.5 ppm resulted in early vaginal opening. Elevated serum LH was also observed in females exposed to

10 ppb arsenic *in utero* suggesting the animals may have enter puberty precociously. Despite these reproductive anomalies, fertility of the exposed females was not significantly different from controls.

Impacts of in utero arsenic exposure on body weight, body composition, and glucose metabolism

The increased body weight during puberty (Figure 2) prompted us to ask whether this weight increase continues later in life and affects metabolism. We followed body weight changes from 3 to 15 weeks of age in females exposed to arsenic *in utero* (Figure 4A). The body weights of both 10 ppb and 42.5 ppm treatment groups were significantly higher than the controls at all time points, particularly after 5 weeks of age. The percentage of fat vs. lean mass measured by Piximus Scans in adult females at 4.5 months of age was significantly higher in both 10 ppb and 42.5 ppm groups (Figure 4B, 10 ppb: 30.6 ± 3 %; 42.4 ppm: 34.1 ± 2% vs. Control: 22.3 ± 2%; $P< 0.05$). In addition to higher body weight and body fat content, the 10 ppb and 42.5 ppm groups also showed signs of impaired glucose tolerance. Forty minutes following glucose challenge, the serum glucose level in the control group started to decline whereas the level remained significantly higher in the 10 ppb and 42.5 ppm groups and did not start to decline until the 60 minute time point (Figure 4C). It is worth noting that serum glucose levels before the challenge (time zero) and 180 min after the challenge were not different among groups, indicating that the treated animals were not diabetic.

At 6 months of age, the body weights of females exposed to 10 ppb arsenic *in utero* remained higher than controls (Figure 5A; 41.9 ± 2.7 vs. 34.4 ± 2.3 g respectively; $P<0.05$). Serum levels of leptin and insulin, two hormones associated with metabolic syndrome and obesity (reviewed by (Fellmann et al. 2013), showed a tendency to be elevated in the 10 ppb

group compared to control (Figure 5B & 5C, p=0.11 for leptin and p=0.06 for insulin) and were statistically significant between control and 42.5 ppm (Figure 5B & 5C, p=0.05 for leptin and p=0.03 for insulin). In summary, females exposed to arsenic *in utero* at 10 ppb and 42.5 ppm became obese starting in young adulthood, probably due to an increase in body fat deposition. Furthermore, exposed females exhibited glucose intolerance compared to the controls. At 6 months of age, the increased body weights of exposed females were still apparent with tendencies of higher levels of circulating leptin and insulin.

Discussion

Most of the animal studies on the health impact of arsenic exposures focus primarily on the high doses (ppm level) with exposure period in the adulthood. Analyses of arsenic exposure during fetal life indicate that the exposed mice are more susceptible to tumors than controls (Tokar et al. 2010; Waalkes et al. 2004a; Waalkes et al. 2006; Waalkes et al. 2007). However, the consequence of exposure on other physiological functions remains unclear. In our study, we investigated the impact of *in utero* exposure to EPA drinking water standard (10 ppb) and a tumor-inducing (42.5 ppm) level of arsenic on reproductive and metabolic functions when the exposed females reach adulthood. We focused specifically on the window of organ formation (E10 to birth). Unexpectedly, both 10 ppb and 42.5 ppm exposure resulted in early vaginal opening, an indicator of puberty, and increased body weight compared to controls. The pattern of increase in body weight and fat composition is similar to the changes observed in the high fat diet-induced obesity model in CD-1 female adult mice (Gao et al. 2015; Lei et al. 2007). Therefore we consider the exposed females in our study obese. While the obese-inducing high fat diet was introduced in the adulthood, in our study the potential obesity-inducing agent (arsenic) was only given during fetal life. These observations, along with a tendency for an

increase in circulating levels of leptin and insulin, suggest that exposure to arsenic during the developmental window from E10 to birth *in utero,* even at levels as low as 10 ppb, could be a contributing factor for obesity and metabolic syndrome in adult female mice.

To our knowledge, effects of *in utero* exposure to low levels of arsenic on age of vaginal opening in mice have never been reported. However, arsenic exposure, particularly at the ppm level, has been linked to delayed puberty in other species. In rats, exposure to 10 mg/kg of arsenic in the drinking water from 12 days of age to puberty led to delayed sexual maturity (Reilly et al. 2014). Delayed puberty was also observed in rats exposed to 3 ppm arsenic *in utero* to 4 months of age (dams were exposed to arsenic prior to breeding and throughout gestation) (Davila-Esqueda et al. 2012). These observations suggest that arsenic exposure at high levels during the peripubertal period may delay reproductive maturity or onset of puberty. The differences in dosing level, time of exposure, and species could contribute to the opposing outcome in our study.

Although early onset of vaginal opening was observed in both 10 ppb and 42.5 ppm groups, the mechanism underlying this phenotype appears to be different. The negative correlation between body weight and age at vaginal opening was maintained in both control and 42.5 ppm groups as expected. However, the correlation between body weight and onset of vaginal opening was not observed in females exposed to 10 ppb. The lack of correlation between body weight and onset of vaginal opening in the 10 ppb group indicates that increased body weight probably does not contribute to precocious puberty at this exposure level. We speculate that our *in utero* exposure paradigm could have a specific impact on the development of the hypothalamic-pituitary-gonadal axis. Age of vaginal opening and levels of circulating LH have been used as indicators of puberty in mice (Risma et al. 1997). Elevated serum LH, which is

positively linked to precocious puberty (Risma et al. 1997), was observed only in the 10 ppb exposed females. In addition to LH, ovary-derived estrogen is also involved in onset of vaginal opening. The levels of estrogen during the prepubertal period vary from day to day with a significant decrease on the day of vaginal opening and an increase 2 -3 days afterwards (Safranski et al. 1993). We examined the serum estradiol level at 21 and 28 days of age (regardless of status of vaginal opening) and found that the majority of animals had undetectable levels of estradiol. Therefore we were not able to establish a connection between estrogen level and early onset of vaginal opening. Although under our experimental conditions we were unable to detect fertility differences between the control and treatment groups, we observed that females in the 42.5 ppm group had fewer litters, fewer pups per litter and a shorter fertile period. These data suggests that exposure to the 42.5 ppm has a more dramatic negative effect on fertility compared to 10 ppb exposure. More studies are needed to better understand this phenotype.

The arsenic-induced weight gain was not restricted to prepubertal mice in our study. The significant weight increase continues in adulthood, accompanied by higher body fat content in both 10 ppb and 42.5 ppm groups compared to controls. One of the contributing factors for obesity in adulthood is low birth weight (Beauchamp et al. 2015). To determine whether the weight increase in exposed female is the result of low birth weight, we measured body weights of exposed embryos at E18, one day before birth. We found that following exposure to 10 ppb, body weight of E18 fetuses was actually increased compared to control and no differences were detected between controls and 42.5 ppm exposed embryos (see Supplemental Figure S1C). These data suggest that the obesity observed in the exposed mice is not related to lower birth weights. A study that tested a single exposure of arsenic (5 mg/kg) at E8 in C57BL6/J mice showed a significant increase in fetal body weight at E18 (Machado et al. 1999). Interestingly in another

study, the birth weight of C57BL6/J female pups exposed to 10 ppb arsenic from E8 to birth was not different from controls and no differences in weight were observed when they reached 8 weeks of age (Ramsey et al. 2013b). Furthermore, C57BL6/J mice exposed to 10 ppb arsenic during the entire pregnancy exhibited no difference in body weight gain at weaning compared to the control (Kozul-Horvath et al. 2012). The different response to 10 ppb arsenic exposure *in utero* between CD-1 (our study) and C57BL6/J strains (Kozul-Horvath et al. 2012; Ramsey et al. 2013a) highlights the potential involvement of genetic backgrounds. Numerous studies have reported the differences among different mouse strains in sensitivity of exposures to various chemicals (Bowen et al. 2010; Kimura et al. 2005; Robinson et al. 2010; Yan et al. 2011). It is generally accepted that C57BL6/J and related strains exhibit a higher sensitivity to arsenic and cadmium exposures based on developmental malformations (Hovland et al. 1999; Machado et al. 1999; Ramsey et al. 2013a). The differences in the window and route of exposure along with genetic backgrounds could contribute to variability of the outcomes following *in utero* arsenic exposure.

In addition to the incidence of obesity, glucose intolerance was observed in female mice exposed to 10 ppb and 45.5 ppm arsenic *in utero*. It was reported that adult mice exposed to arsenic at 50 ppm or higher developed impaired glucose tolerance (Hill et al. 2009; Paul et al. 2011). A recent study concluded that exposure of adult mice to 3 mg/L (3 ppm) sodium arsenite for 16 weeks resulted in altered glucose metabolism and pancreatic function (Liu et al. 2014). In human adults, chronic arsenic exposure (greater than>100 ppb) was associated with diabetes (Islam et al. 2012; Tseng et al. 2000). Type 2 diabetes was also correlated with low to moderate levels of arsenic exposure (Navas-Acien et al. 2008). Under our experimental conditions, the significantly higher body weights observed in the 10 ppb exposed animals were maintained

through 6 months of age (a trend was observed for the 42.5 ppm exposed animals). A trend for higher circulating levels of leptin and insulin was also detected in the arsenic exposed groups. We suspect that under our experimental conditions, *in utero* arsenic exposure could cause permanent alteration in lipid metabolism, leading to obesity phenotypes such body fat increase and glucose intolerance (Cheng et al. 2011; Kozul-Horvath et al. 2012). Obesity is associated with leptin resistance (reviewed in (Myers et al. 2010) and the trend to higher circulating levels of leptin in arsenic-exposed mice could be a potential mechanism that deserves further investigation.

Although our findings reveal perturbation of puberty onset, obesity, and glucose metabolism induced by *in utero* arsenic exposure, the mechanism of arsenic action underlying these changes remains unknown. Most effects of arsenic exposure in adults are attributed to the activation of gene pathways that increase reactive oxygen species and oxidative stress (Ghatak et al. 2011; Kitchin and Ahmad 2003; Lu et al. 2014). Changes in DNA methylation were reported in C57BL6/J adult mice in the lung following exposure for 90 days to 50 ppm arsenic (Boellmann et al. 2010). Changes in methylation status in liver were also found in adult C57BL6/J mice after 5 months of exposure to 50 ppm arsenic in drinking water (Nohara et al. 2011). Methylation changes in human cord blood have also been associated with *in utero* arsenic exposure (Koestler et al. 2013). Given that the exposure window of our study is restricted to the second half of fetal life, the adult onset of perturbations could derive from epigenetic changes as a consequence of *in utero* arsenic exposure. These epigenetic changes may have impacts at the cellular and/or systemic level that alter metabolism and hormone production.

The most interesting aspect of our results is that *in utero* exposure to 10 ppb arsenic, the EPA approved drinking water level, causes similar or even more detrimental effects on body

weight and age of vaginal opening than the tumor-inducing 42.5 ppm level. In CD-1 mice arsenic appears to have different cellular effects dependent upon the dose and time of exposure. Further studies are needed to understand the potential mechanisms underlying the action of arsenic in a dose-dependent manner.

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Table 1: Figure assignment, endpoints of measurement, and sample sizes

Figure	Endpoints	Sample size
Figure 2	Vaginal opening detection from 18-29 days of age Body weight measurement to 8 weeks of age	Control: 29 10 ppb: 37 42.5 ppm: 35
Figure 3	LH and FSH and Estradiol measurement	Control: 5-6 10 ppb: 5-6 42.5 ppm: 4-8
Figure 4A	Body weight measurement from 9 to 15 weeks of age	Control: 17 10 ppb: 25 42.5 ppm: 21
Figure 4B	Body fat composition measurement at 4.5 months of age	Control: 9 10 ppb: 10 42.5 ppm: 14
Figure 4C	Glucose tolerance assay at 5 months of age	Control: 7 10 ppb: 10 42.5 ppm: 11
Figure 5	Body weight and measurement of leptin and insulin at 6 months of age	Control: 5 10 ppb: 7 42.5 ppm: 6
Table 2	Analysis of estrous cyclicity for 18 days starting at 10 weeks of age	Control: 17 10 ppb: 22 42.5 ppm: 21
Table 3	Fertility analysis: number of litters, total number of pups per litter, total number of pups per female, days between litters, and fertile period	Control: 5 10 ppb: 8 42.5 ppm: 13
Sup. Figure 1A&B	Maternal weight gain and litter size	Control: 7 10 ppb: 7 42.5 ppm: 7
Sup. Figure 1C	Fetal body weight at E18	Control: 46 10 ppb: 30 42.5 ppm: 38
Sup. Figure 2	Ovarian histology at 21 days, 28 days, and 6 months of age	Control: 5 10 ppb: 7 42.5 ppm: 6
Sup. Figure 3	Serum level of estradiol, testosterone, progesterone, and DHEA at 6 months and 1 years of age	Control: 5 10 ppb: 7 42.5 ppm: 6

Table 2: Percentage of the time that the female spent in each stage of the estrous cycle

Endpoint	Controls (n=17)	10 ppb (n=22)	42.5 ppm (n=21)	p-value
% in estrus	28.1 ± 2.9	28.8 ± 2.9	27.8 ± 2.2	0.962
% in metestrus	31.0 ± 1.4	30.0 ± 2.0	25.9 ± 1.8	0.114
% in diestrus	20.6 ± 1.8	24.7 ± 2.2	28.0 ± 2.7	0.092
% in proestrus	20.3 ± 1.4	16.4 ± 1.8	18.2 ± 1.5	0.267

Numbers represent averages ± standard error.

Table 3: Effects of *in utero* arsenic exposure on fertility of adult females

Treatment	Days to 1st litter	Days between litters	Number of pups per litter	Total pups produced per female	Total number of litters per female	Fertile period (days)
Control (n=5)	21.6 ± 0.40	29.04 ± 1.44	13.65 ± 0.93	112.80 ± 12.04	9.00 ± 1.30	254.4 ±18.6
10 ppb (n=8)	22.00 ± 1.62	28.55 ± 1.53	11.03 ± 1.08	124.88 ± 8.56*	10.38 ± 0.94*	302.3 ± 16.5*
42.5 ppm (n=13)	23.31 ± 1.01	31.65 ± 1.59	12.32 ± 0.43	73.77 ± 13.38*	5.77 ± 1.04*	186.1 ± 30.62*

Numbers represent averages ± standard error.
* represents significant differences between 10 ppb and 42.5 ppm groups with $p<0.05$. There are no differences between treatment groups and the Control.

Figure Legends

Figure 1: Experimental design: pregnant CD-1 females were exposed to 0 (control), 10 ppb, or 42.5 ppm of sodium arsenite in drinking water from E10 to birth. At birth, pups were fostered to females that were not exposed to arsenic. Vaginal opening was checked daily starting at 18 days of age and the body weight was recorded weekly for 15 weeks. At 8 weeks of age, female pups were set up for fertility test or analyzed for metabolic endpoints. Sample size for each experiment is listed in Table 1.

Figure 2: Effects of *in utero* arsenic exposure on onset of vaginal opening and body weight. (A) X axis represents the day of vaginal opening. Y axis represents the percentages of animals with open vagina. (B) Y axis represents the average age ± SE when vaginal opening was observed. * indicates $P<0.05$. (C) Average body weight at 21 days of age (average age ± SE); (D) lines represent correlations between body weights (BW) and onset of vaginal opening (VO).

Figure 3: Effects of *in utero* arsenic exposure on levels of serum LH (A & B) and FSH (C & D) at 21 and 28 days of age (mean ± SE). * indicates $P<0.05$ compared to control group.

Figure 4: Effects of *in utero* arsenic exposure on (A) body weight from 3 weeks to 15 weeks of age. * indicates significant difference ($P< 0.05$) between the control and 10 ppb group and + indicates significant difference ($P<0.05$) between control and 42.5 ppm group. (B) percentage body fat. * indicates significant difference ($P<0.05$) compared to control, and (C) glucose tolerance analysis. Y axis represents serum glucose levels (mean ± SE, * indicates $P<0.05$ compared to control).

Figure 5: Effects of *in utero* arsenic exposure on (A) body weight, (B) serum leptin, and (C) serum insulin level at 6 months of age (Means ± SE; * indicates $P< 0.05$ compared to control).

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Figure 1

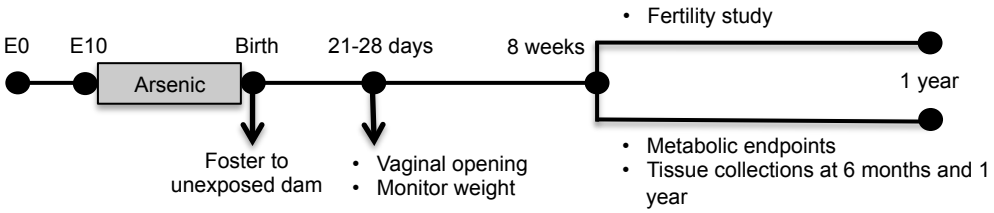

Figure 2

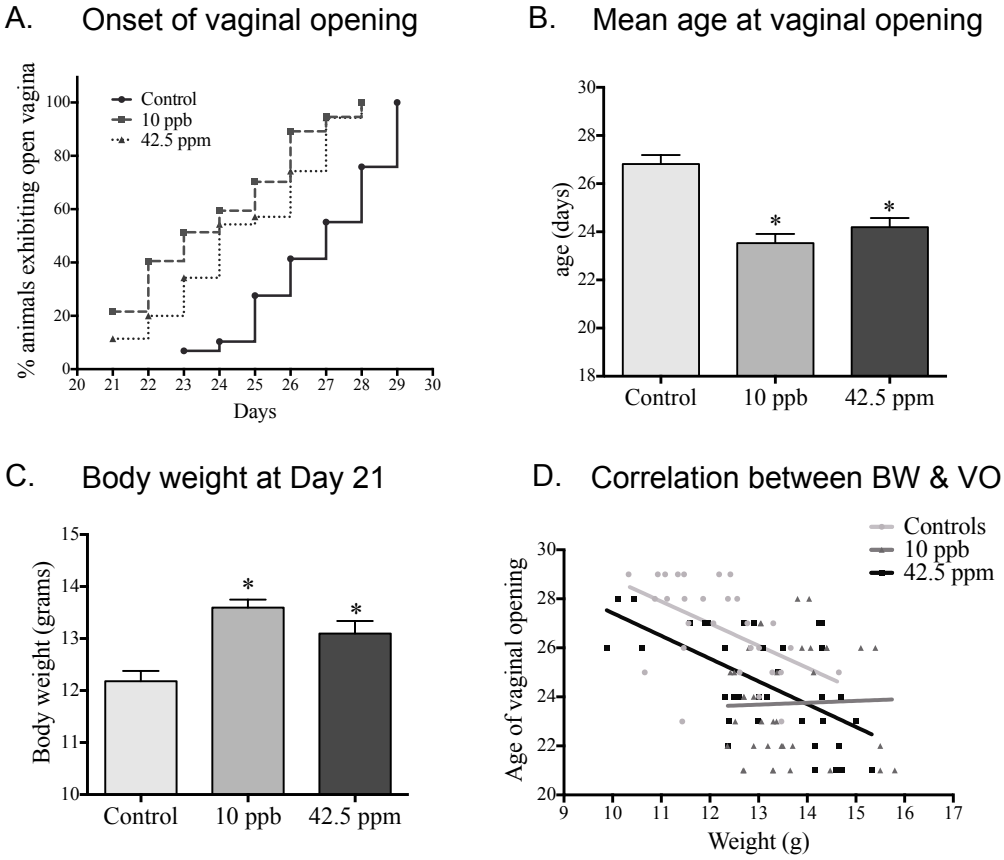
A. Onset of vaginal opening
% animals exhibiting open vagina
Control
10 ppb
42.5 ppm
Days
B. Mean age at vaginal opening
age (days)
Control
10 ppb
42.5 ppm
C. Body weight at Day 21
Body weight (grams)
Control
10 ppb
42.5 ppm
D. Correlation between BW & VO
Age of vaginal opening
Weight (g)
Controls
10 ppb
42.5 ppm

Figure 3

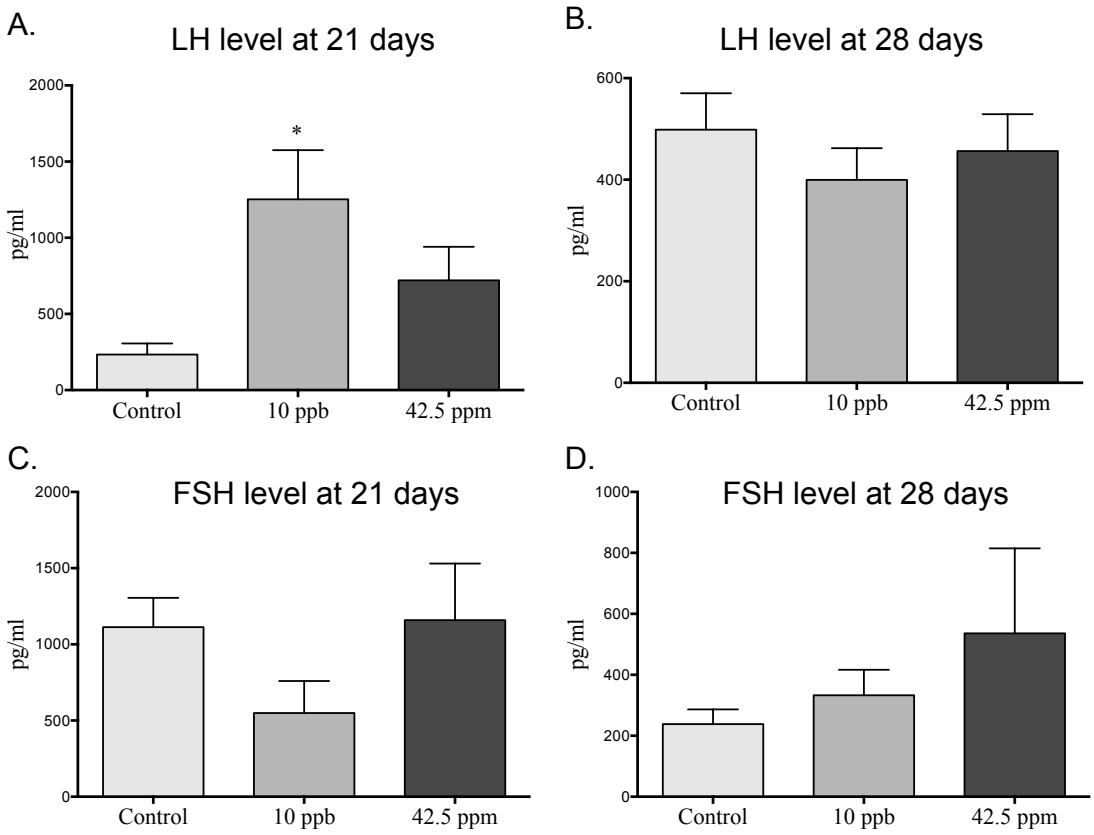
A.
LH level at 21 days
pg/ml
2000
1500
1000
500
0
*
Control
10 ppb
42.5 ppm
B.
LH level at 28 days
pg/ml
600
400
200
0
Control
10 ppb
42.5 ppm
C.
FSH level at 21 days
pg/ml
2000
1500
1000
500
0
Control
10 ppb
42.5 ppm
D.
FSH level at 28 days
pg/ml
1000
800
600
400
200
0
Control
10 ppb
42.5 ppm

Figure 4

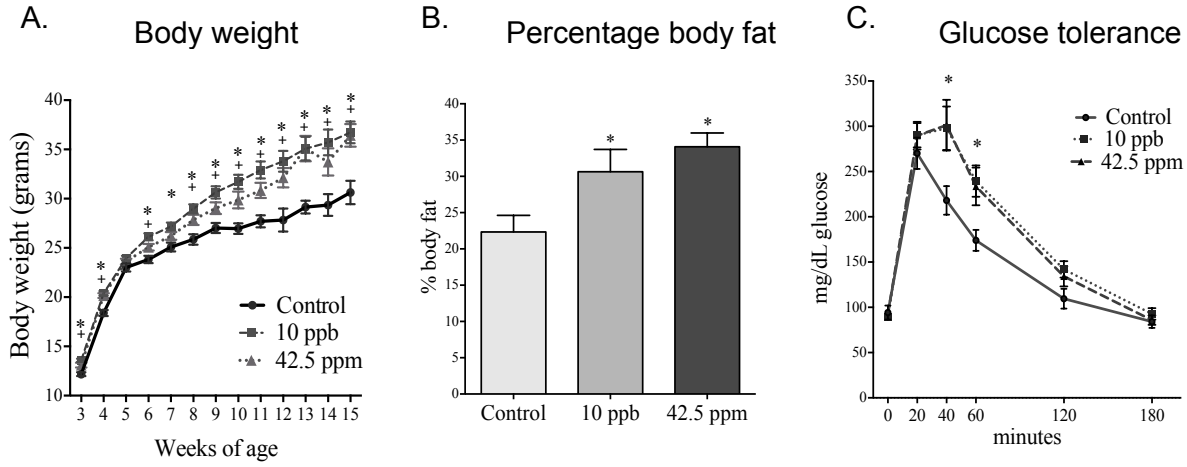
A.
Body weight
Body weight (grams)
Weeks of age
Control
10 ppb
42.5 ppm
B.
Percentage body fat
% body fat
Control
10 ppb
42.5 ppm
C.
Glucose tolerance
mg/dL glucose
minutes
Control
10 ppb
42.5 ppm

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Figure 5